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Selectable marker-free transgenic plants without sexual crossing: transient expression of *cre* recombinase and use of a conditional lethal dominant gene

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Abstract

Transgenic tobacco plants were produced that contained single-copy pART54 T-DNA, with a 35S-*uid*A gene linked to *lox*P-flanked kanamycin resistance (*npt*II) and cytosine deaminase (*cod*A) genes. Retransformation of these plants with pCre1 (containing 35S transcribed *cre* recombinase and hygromycin (*hpt*) resistance genes) resulted in excision of the *lox*P-flanked genes from the genome. Phenotypes of progeny from selfed-retransformed plants confirmed *npt*II and *cod*A excision and integration of the *cre*-linked *hpt* gene. To avoid integration of the *hpt* gene, and thereby generate plants totally free of marker genes, we attempted to transiently express the *cre* recombinase. *Agrobacterium tumefaciens* (pCre1) was cocultivated with leaf discs of two pART54-transformed lines and shoots were regenerated in the absence of hygromycin selection. Nineteen of 773 (0.25%) shoots showed tolerance to 5-fluorocytosine (5-fc) which is converted to the toxic 5-fluorouracil by cytosine deaminase. 5-fc tolerance in six shoots was found to be due to excision of the *lox*P-flanked region of the pART54 T-DNA. In four of these shoots excision could be attributed to *cre* expression from integrated pCre1 T-DNA, whereas in two shoots excision appeared to be a consequence of transient *cre* expression from pCre1 T-DNA molecules which had been transferred to the plant cells but not integrated into the genome. The absence of selectable marker genes was confirmed by the phenotype of the T_1 progeny. Therefore, through transient *cre* expression, marker-free transgenic plants were produced without sexual crossing. This approach could be applicable to the elimination of marker genes from transgenic crops which must be vegetatively propagated to maintain their elite genotype.

Abbreviations: BAP, 6-benzylaminopurine; CaMV 35S, cauliflower mosaic virus 35S promoter; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; NAA, 1-naphthaleneacetic acid; ORF, open reading frame; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; SV40, simian virus 40; UTR, untranslated region; X-Gluc, 5-bromo-4-chloro-3-indolyl glucuronide

Introduction

As part of the process of plant transformation dominant selectable markers are used to select transgenic cells, from which intact transgenic plants can be regenerated. These marker genes are generally superfluous once an intact transgenic plant has been established. Antibiotic or herbicide resistance genes account for the majority of selectable markers used (Yoder and Goldsbrough, 1994) and their presence in transgenic crop plants has generated a number of environmental and consumer concerns (Zechendorf, 1994). Risk assessment reports have argued that there are no scientific, health or safety reasons to restrict the use of the neomycin phosphotransferase (*npt*II) selectable marker gene (Fuchs *et al.*, 1993; Nap *et al.*,

1992). However, this may not be true of other selectable markers and necessitates a lengthy and expensive risk assessment process on each marker gene and its product. This could cause considerable delays in the release of a transgenic crop. Furthermore, regardless of the assurances provided in risk assessment reports, it is consumer acceptance of a product that governs its market performance. Therefore it would be sensible to alleviate perceived risks by eliminating the marker genes.

Commercial crop cultivars developed through genetic engineering are likely to contain several distinct traits introduced into an established cultivar. Multiple novel traits could be brought together in an elite cultivar by conventional sexual crossing. However, for perennial fruit crops this is not ideal because of their long generation time. Furthermore, sexual crossing results in the loss of the desirable combination of existing traits due to recombination. Repeated transformation to sequentially introduce each novel trait would allow maintenance of the elite genotype and avoid the long time before plants can be crossed. However, the presence of a functional marker gene precludes its use in subsequent transformations and only a limited number of selectable marker genes have proved to be useful in crops recalcitrant to transformation (Dekeyser *et al.*, 1989; Zhou *et al.*, 1995), including apple and kiwifruit which are the subject of much work in our laboratory. Therefore, removing the selectable marker gene from a transgenic plant would allow the optimal selection procedure to be used repeatedly in subsequent transformations.

Several strategies have been employed to generate marker-free transgenic plants (Yoder and Goldsbrough, 1994). These include: transposition-mediated repositioning of the marker gene (Goldsbrough *et al.*, 1993); co-transformation of two T-DNA molecules (Komari *et al.*, 1996; McKnight *et al.*, 1987) and site-specific recombination (Dale and Ow, 1991; Russell *et al.*, 1992). As each of these strategies require sexual crossing to eliminate all the marker genes their applicability to plant species which have a long generation time is somewhat limited. The only strategy currently available which can generate marker-free transgenic plants without the need to sexually cross plants is the MAT vector system (Ebinuma et al., 1997).

Here we describe plant transformation vectors, incorporating the Cre/*lox*P site-specific recombination system to facilitate the elimination of marker genes from transgenic plants. Site-specific recombinases of the λ integrase family have been used to manipulate DNA in heterologous cellular environments (reviewed in Kilby *et al.*, 1993; Odell and Russell, 1994; Sauer, 1993). The *Escherichia coli* bacteriophage P1 Cre/*lox*P, *Zygosaccharomyces rouxii* R/*rs* and *Saccharomyces cerevisiae* Flp/*frt* recombination systems require only the recombinase and target sequences for recombination and have been shown to function in plants (Dale and Ow, 1990; Kilby *et al.*, 1995; Onouchi *et al.*, 1991). The 38 kDa Cre recombinase can interact with two 34 bp *lox*P sites and when present in a direct repeat orientation result in excision of the intervening DNA (Bayley *et al.*, 1992; Dale and Owe, 1990, 1991; Odell *et al.*, 1990; Russell *et al.*, 1992). Our strategy also utilises a conditional lethal dominant gene, which allows a normally non-toxic compound to be converted to a toxic agent. This gene, which has been described for use in plants, is the *E. coli* cytosine deaminase (*cod*A) (Perera *et al.*, 1993; Stougaard, 1993) whose product converts 5-fluorocytosine to 5-fluorouracil, a precursor of 5-fluoro-dUMP which irreversibly inhibits thymidylate synthase activity and consequently deprives the cells of dTTP for DNA synthesis.

Here we report on the transient expression of the *cre* recombinase to mediate excision of *lox*P-flanked marker genes from transgenic plants and the use of the *cod*A gene to select plants, which have undergone Cre-mediated recombination. We demonstrate that using this approach, marker-free transgenic plants can be produced without sexual crossing.

Materials and methods

The pART53 and pART54 binary vectors

DNA manipulations were performed essentially as described in Sambrook *et al.* (1989). We constructed the vector pART53 for *Agrobacterium*-mediated plant transformation. The pART53 backbone is based on pART27 (Gleave, 1992) with the RK2 minimal replicon and ColE1 origin of replication for maintenance in *Agrobacterium* and *E. coli*, respectively, and the Tn7 spectinomycin/streptomycin resistance gene for bacterial selection. The pART53 T-DNA consists of the right T-DNA border followed by a unique *Not*I site. Adjacent to the *Not*I site is a *lox*P recombination sequence followed by a chimeric kanamycin (*npt*II) resistance gene (nopaline synthase promoter-neomycin phosphotransferase-nopaline synthase 3′ UTR). Ad-

jacent to the chimeric *npt*II gene is a chimeric cytosine deaminase (*cod*A) gene (35S-*E. coli cod*A ORF-octopine synthase 3′ UTR) and immediately 3′ of this chimeric *cod*A gene is a *lox*P recombination sequence in the same orientation as the one upstream of the chimeric *npt*II gene. Thus *lox*P sequences flank the chimeric *npt*II and *cod*A genes. The left T-DNA border is adjacent to the second *lox*P sequence. A 35S-*uid*A-*ocs* 3′ cassette was cloned as a 4.2 kb *Not*I fragment into pART53, in an orientation that placed the 35S promoter adjacent to the right T-DNA border. This vector was designated pART54 (Figure 1A). Precise vector construction details can be obtained from the authors on request.

The pCre1 binary vector

The *cre* recombinase gene was obtained from pBS7 (Sauer, 1987). PCR amplification was carried out on pBS7 with primers Cre1 (5′-GCCGCTCGAGGTACC-ATGGCTCCAAAGAAGAAGAGAAAGGTTGAAG-ACCCACGC**ATGTCCAATTTACGTACCGTA**-3′) and Cre2 (5′-GCCGGAATTCAAGCTT**ATCAACTA-ATTATAGCAATC**-3′). Primer Cre1 anneals to the *cre* ORF at nucleotide positions 1–21 (bold) and amplification using this primer results in the translational fusion of the 13 amino acid nuclear localization signal of the SV40 T-antigen (underlined) to the N-terminus of the Cre protein. Primer Cre2 anneals 17–37 nucleotides downstream of the *cre* ORF (bold). Amplifications were carried out on 10 ng of template, with 0.5 mM of each primer, 80 mM dNTPs, 1× *Taq* DNA polymerase buffer, 0.5 U Expand High Fidelity *Taq* DNA polymerase (Boehringer Mannheim), in an Ericomp Cycler: 94 °C (3 min), followed by 25 cycles of 94 °C (30 s), 55 °C (30 s), 72 °C (2 min). The 1.1 kb PCR product was cloned as a *Xho*I-*Hin*dIII fragment into pART7 (Gleave, 1992), placing *cre* under the transcriptional control of the 35S promoter. The 35S-*cre-ocs* 3′ cassette was cloned as a 3.15 kb *Not*I fragment into the binary vector pBJ41 (a pART27-based vector with a 35S-*hpt* gene for hygromycin selection in plants; B. Janssen, unpublished). The resulting binary vector was designated pCre1 (Figure 1B).

Plant transformation

Binary vectors were transformed into electrocompetent *Agrobacterium tumefaciens* LBA4404 using a Cell-Porator apparatus (Gibco-BRL), and selected on spectinomycin (100 mg/l). Transgenic *Nicotiana tabacum* cv. Samsun plants were produced using the leaf-disc transformation procedure (Horsch *et al.*, 1985) but without nurse cells and were selected on either kanamycin (100 mg/l) or hygromycin (10 mg/l). Individual plantlets were maintained in tissue culture under artificial light (16 h light/8 h dark) at 25 °C, and clonally propagated to provide material for molecular analysis, histochemical staining and *cod*A expression screening, or were transferred to a containment glasshouse facility and maintained with supplementary lighting (16 h light/8 h dark) at 20–30 °C, until seed set. T_1 seeds were surface-sterilised and germinated on ½ MS medium containing 250 mg/l kanamycin or 15 mg/l hygromycin.

Screening for codA *expression and 5-fluorocytosine treatment of regenerated shoots*

Clonally propagated plants were transferred to MS medium containing 5-fluorocytosine (1 g/l). Plants were maintained in tissue culture under artificial light (16 h light/8 h dark) at 25 °C for 2–3 weeks after which time they were scored for *cod*A expression on the basis of plant survival or death. Progeny of T_0 plants were screened for *cod*A expression by plating surface-sterilised seeds onto ½ MS medium containing 5-fluorocytosine (500 mg/l). Seeds were maintained under artificial light (16 h light/8 h dark) at 25 °C. Failure of seeds to germinate was indicative of *cod*A expression.

After cocultivation with *A. tumefaciens* LBA4404 (pCre1), leaf discs were placed onto MS medium for 48 h then transferred to MS medium containing NAA (0.1 mg/l), BAP (1 mg/l) and cefotaxime (100 mg/l). Regenerating shoots were transferred to hormone-free MS medium and within 2 days of showing root initiation were transferred to hormone-free MS medium containing 5-fluororocytosine (500 mg/l), and maintained for 2–3 weeks. Surviving plantlets were transferred to fresh hormone-free MS medium.

Southern analysis of transgenic plants

Genomic DNA was extracted from 100 mg of leaf tissue using the CTAB procedure (Doyle and Doyle, 1990), digested, electrophoresed through 0.7% TBE agarose gels, depurinated (0.25 M HCl) and transferred onto Hybond N^+ nylon membrane (Amersham) following the manufacturer's recommendations for alkaline transfer. Membranes were prehybridised for 6 h at 65 °C in 10 ml of 500 mM Na_2HPO_4 pH 7.2, 1 mM EDTA, 7% SDS, 100 mg/l denatured salmon sperm DNA, followed by hybridisation at 65 °C for 16 h with

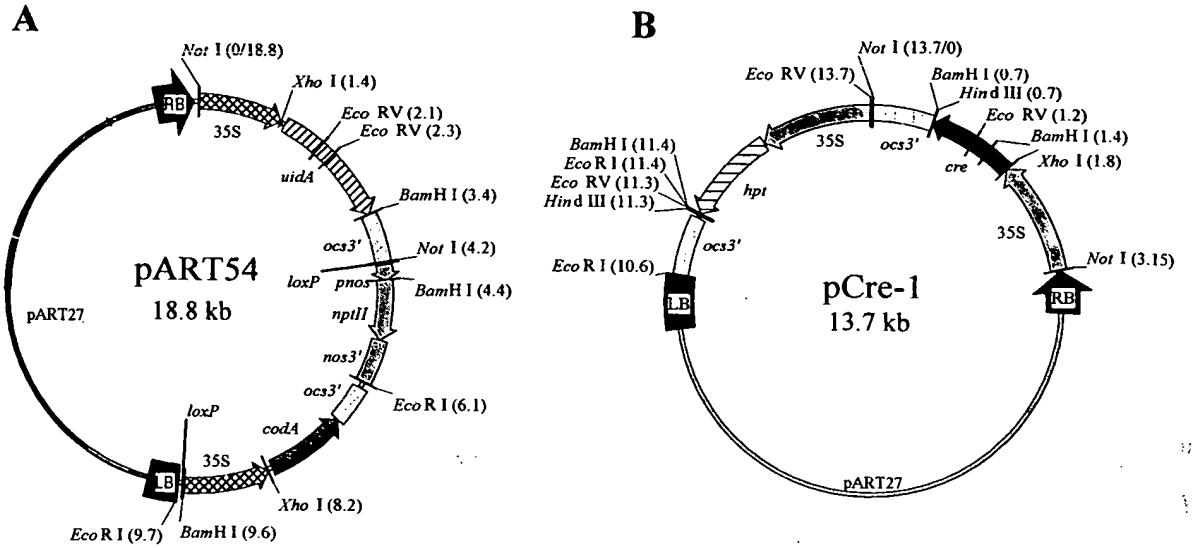

Figure 1. The pART54 and pCre1 binary vectors. **A.** pART54 vector containing a 35S-*uid*A-*ocs*3′ reporter gene, a p*nos*-*npt*II-*nos*3′ gene for kanamycin selection and a 35S-*cod*A-*ocs*3′ gene for 5-fc-negative selection and with the latter two genes flanked by *lox*P sequences. Right (RB) and left (LB) borders delimit the T-DNA and the vector backbone is from pART27. **B.** pCre1 vector containing a 35S-*hpt*-*ocs*3′ gene for hygromycin selection and a 35S-*cre*-*ocs*3′ gene between the right (RB) and left (LB) T-DNA borders. *Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII, *Not*I and *Xho*I sites are indicated.

denatured radioactive DNA probes. Probes were prepared from DNA fragments eluted from agarose gels using Bresa-Clean (Bresatec), and labelled (40 ng) using the Megaprime DNA Labeling System (Amersham) incorporating [α-^{32}P] dCTP. After hybridisation, membranes were washed in 4× SSC (0.6 M NaCl, 60 mM sodium citrate), at 25 °C (15 min), followed by washes in 1× SSC, 0.1% SDS at 65 °C (15 min) and 0.1× SSC, 0.1% SDS at 65 °C (15 min). Hybridisation signals were detected on a Molecular Dynamics Storm 840 phosphoimager after 6–24 h.

Histochemical analysis for β-glucuronidase expression

Detached leaves or seedlings were vacuum-infiltrated for 3–5 min at −60 kPa with 50 mM sodium phosphate pH 7.0, 0.06% Triton X-100, 1% DMSO, 0.5 g/l X-Gluc (dissolved in dimethyl formamide) and incubated overnight at 37 °C. Chlorophyll was removed by bleaching in 100% ethanol.

Results

Production and characterisation of pART54 transgenic tobacco plants

Agrobacterium tumefaciens LBA4404 (pART54) was used to transform *Nicotiana tabacum* cv. Samsun and 20 independent plants, referred to as 5401 to 5420, were regenerated in the presence of kanamycin. Nineteen of these lines were found to express the *uid*A transgene (GUS-positive). Clonally propagated plants of sixteen lines showed stunted root development, chlorosis of the leaves within 14 days followed by a high degree of necrosis and death on media containing 5-fluorocytosine (5-fc), indicating expression of the *cod*A transgene. Healthy growth of 5401, 5406 and 5417 in the presence of 5-fc indicated that the *cod*A transgene was not expressed in these lines.

In the presence of the Cre recombinase, multi-copy pART54 T-DNA inserts containing *lox*P sequences could result in chromosomal deletions, inversions or translocations. Therefore we wished to identify plants with single-copy pART54 T-DNA. Southern analysis on *Eco*RV- and *Bam*HI-digested DNA extracted from each of the 16 GUS-positive, *cod*A expressing pART54 lines and probed with the 5′ end of the *uid*A gene revealed that six of these lines contained single-copy T-DNA inserts (data not shown). Two of these

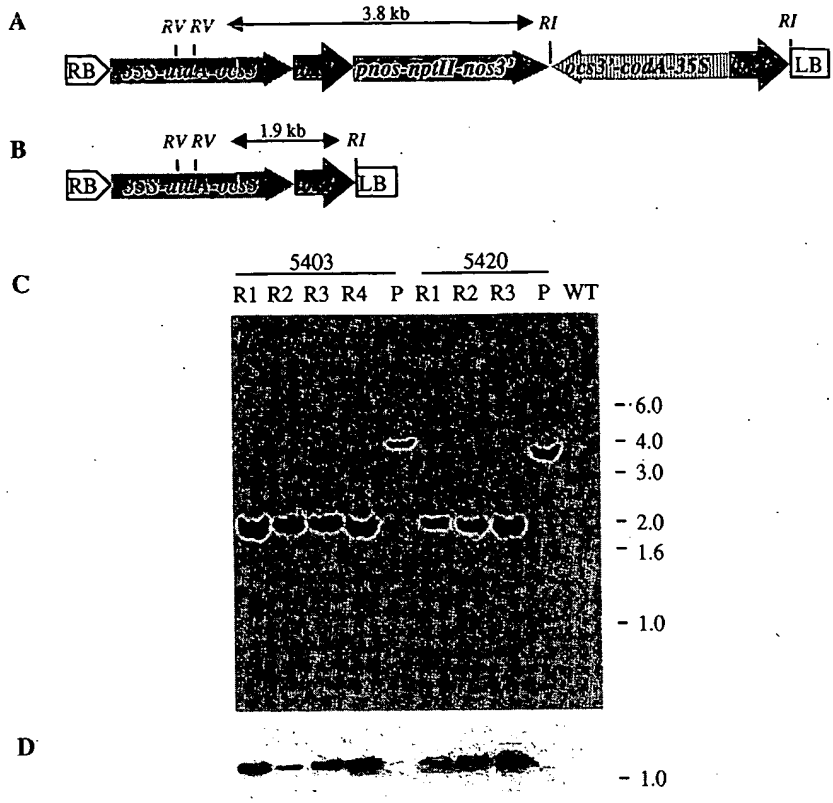

Figure 2. Excision of *lox*P-flanked region of pART54 T-DNA after retransformation with pCre1. **A.** Schematic presentation of the pART54 T-DNA integrated into the genome. **B.** Expected pART54 T-DNA structure after Cre-mediated recombination. *Eco*RI and *Eco*RV sites and fragments which hybridise to the *uid*A 3′ probe are shown. **C.** Southern blot of *Eco*RI/*Eco*RV-digested DNA from 5403 and 5420 parental lines (P) and from four 5403-derived lines (R1–R4) and three 5420-derived lines (R1–R3) regenerated after pCre1 transformation. The membrane was probed with a 1.1 kb *Eco*RV/*Bam*HI fragment encoding the 3′ end of the *uid*A gene. Positions of 1 kb markers (Gibco-BRL) are indicated. **D.** Region of the Southern blot described above after reprobing with a 0.5 kb *Eco*RV/*Bam*HI fragment encoding the 3′ end of the *cre* gene. Position of 1 kb marker is indicated.

single-copy lines (5403 and 5420) were chosen for further studies.

Stable retransformation of pART54 transgenic lines with pCre1

Transgenic tobacco lines 5403 and 5420 were retransformed with *A. tumefaciens* LBA4404 (pCre1) in order to introduce the *cre* recombinase gene. We selected four 5403 (5403R1–4) and three 5420 (5420R1–3) derived lines that were regenerated in the presence of hygromycin for further study. Southern analysis was carried out on *Eco*RI/*Eco*RV-digested genomic DNA extracted from each of the putatively retransformed lines and the original 5403 and 5420 lines, probing with the 3′ end of the *uid*A gene. A single hybridising 1.9 kb fragment would be expected in lines which had undergone Cre-mediated excision of the *lox*P-flanked region of the pART54 T-DNA, whereas hybridisation to a 3.8 kb fragment would be expected in the original pART54 lines and lines which had not undergone Cre-mediated excision (Figure 2A and B). Results revealed a single 3.8 kb hybridising fragment in 5403 and 5420, whereas a 1.9 kb hybridising fragment was observed in the seven putative pCre1 retransformants (Figure 2C). This result suggested that these lines had been retransformed with pCre1, that *cre* had been expressed and that Cre-mediated recombination had occurred.

When the *Eco*RI/*Eco*RV-digested DNA was reprobed to detect the presence or absence of pCre1 T-DNA (Figure 2D), hybridisation to the 1.2 kb *Eco*RV fragment was detected in 5403R1–4 and 5420R1–3 but not in the two parental lines. This result confirmed that 5403R1–4 and 5420R1–3 were genuine pCre1 retransformants.

It is possible that after Cre-mediated excision from the pART54 T-DNA the resulting circular *npt*II-*cod*A

molecule could reintegrate into the genome at a random position. However, the absence of any hybridisation signal when *Bam*HI-digested genomic DNA from two lines tested (5403R1 and 5420R1) was probed with the 1.7 kb *Bam*HI-*Eco*RI *npt*II-*nos*3′ fragment of pART54 indicated that, at least in these two lines, no such reintegration event had occurred (data not shown).

Progeny analysis of pART54 lines stably retransformed with pCre1

In the presence of kanamycin a 3:1 resistant-to-sensitive ratio was observed for the progeny of selfed 5403 and 5420, as expected for Mendelian inheritance of single-copy pART54 T-DNA. Progeny of three retransformed lines tested (5403R1, 5420R1 and 5420R2) bleached in the presence of kanamycin, confirming that the *npt*II gene had been excised from the genome (Table 1). Progeny of 5403 and 5420 were found to be sensitive to hygromycin, whereas the ratio of hygromycin-resistant to sensitive progeny of 5403R1, 5420R1 and 5420R2 suggested that these lines contained one, two and two unlinked, expressed copies of the pCre1 *hpt* gene, respectively. In the presence of 5-fc 25% of the 5403 and 5420 progeny germinated, as would be expected given that 25% of the seeds would be homozygous and 50% hemizygous for the conditional lethal dominant expressed *cod*A transgene, and that seeds would only germinate in the absence of *cod*A expression. Almost 100% of the progeny of 5403R1, 5420R1 and 5420R2 germinated in the presence of 5-fc, confirming excision of *cod*A. Of these 5-fc-tolerant seedlings, 75% were found to be GUS-positive and therefore retained the *uid*A gene but not the *npt*II and *cod*A marker genes.

Southern analysis of *Eco*RI/*Eco*RV-digested genomic DNA, extracted from four 5-fc-tolerant progeny of both 5420R1 and 5420R2 and probed with the 3′ end of *uid*A, revealed a 1.9 kb hybridising fragment, indicative of pART54 T-DNA, which had undergone excision of the *lox*P-flanked region, in two 5420R1 progeny and three 5420R2 progeny (Figure 3). The remaining 5420R1 (a and b) and 5420R2 (c) progeny showed no hybridising fragments. The presence of this 1.9 kb hybridising fragment correlated with β-glucuronidase activity.

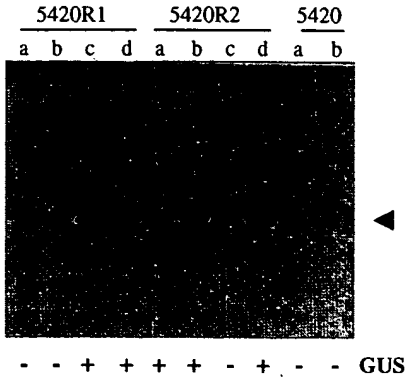

Figure 3. 5-fc-tolerant progeny of pART54/pCre1 plants. Southern blot of *Eco*RI/*Eco*RV-digested DNA from 5-fc-tolerant progeny (a–d) of selfed pART54 (5420) and pART54/pCre1 (5420R1 and 5420R2) plants. The membrane was probed with a 1.1 kb *Eco*RV/*Bam*HI fragment encoding the 3′ end of the *uid*A gene. Arrow indicates position of the 1.9 kb hybridising fragment. Results of histochemical staining of each seedling for β-glucuronidase are shown.

Cocultivation of pART54 transgenic tissue with A. tumefaciens *pCre1 in the absence of selection*

A total of 120 leaf discs from each of transgenic plant lines 5403 and 5420 were cocultivated with *A. tumefaciens* LBA4404 (pCre1) in two separate experiments. In the presence of NAA and BAP numerous shoots were evident after 3–4 weeks. A total of 360 independent shoots from 5403 leaf discs and 413 independent shoots from 5420 leaf discs were transferred to fresh medium. Upon showing root initiation (5–10 days) the shoots were placed under 5-fc selective pressure and under these conditions the majority of these plantlets failed to show root elongation, began to show chlorosis of the leaves within 10 days, followed by necrosis and death within 10–21 days. Nineteen shoots, ten derived from 5403 (5403T1–T10) and nine derived from 5420 (5420T1–T9) did survive the 5-fc selection, although some of these plantlets (5403T1, T2, T9, T10 and 5420T2, T3, T4, T9) did show some degree of chlorosis.

Analysis of 5-fc-tolerant regenerants

*Eco*RI/*Eco*RV-digested genomic DNA extracted from 5403 and 5420 and each of the 19 lines which survived the 5-fc selection were probed with the 3′ end of *uid*A (Figure 4A). A 1.9 kb hybridising fragment, indicative of Cre-mediated excision of the *lox*P-flanked region of pART54 T-DNA, was observed in three 5403-derived lines (5403T4, 5403T8 and 5403T9) and three 5420-derived lines (5420T1, 5420T6 and

Table 1. Phenotype analysis of progeny of selfed pART54 and pART54 plants retransformed with pCre-1.

Line	Genotype[1]	Seed viability[2],%	kan^R 250	hyg^R 15	5-fc^R 0.5	GUS^+/5-fc^R
Wild-type	–	>95	0/107	0/101	52/54	0/26
5403	[*npt*II *cod*A *uid*A]	100	116/146	0/137	30/102	0/30
5403R1	[*uid*A] [*cre-hpt*]	100	0/108	64/84	69/71	36/46
5420	[*npt*II *cod*A *uid*A]	100	112/145	0/182	23/79	0/23
5420R1	[*uid*A] [*cre-hpt*]	100	0/120	101/109	136/136	38/50
5420R2	[*uid*A] [*cre-hpt*]	>97	0/112	127/135	82/83	36/50

[1]Genotype based on Southern analysis.
[2]Germination on ½ MS medium.

5420T7). In addition to the 1.9 kb hybridising fragment, 5403T9 showed a weaker hybridisation signal at 3.8 kb, suggesting that this line was chimeric for the Cre-mediated excision. It was also noted that of the six lines showing evidence of Cre-mediated excision only 5403T9 showed some degree of chlorosis during 5-fc selection. The 3.8 kb hybridising signal in 5403T1, T2, T3, and T10, and 5420T2, T3, T4, T5, T8 and T9 indicated that Cre-mediated excision had not occurred in these lines. The absence of any hybridisation signal in 5403T5, T6 and T7 suggested that the original 5403 line was in fact chimeric for pART54 T-DNA and that these regenerants were derived from cells which did not contain the T-DNA and so in lacking *cod*A were unaffected by the presence of 5-fc. These results indicated that 6 of the 19 plantlets survived 5-fc treatment due to Cre-mediated excision of the *lox*P-flanked *cod*A gene of the pART54 T-DNA.

To establish whether the Cre-mediated excision in these 6 lines could be attributed to expression of *cre* from integrated pCre1 T-DNA or was a consequence of transient expression of *cre* without T-DNA integration, the genomic DNA was probed for the presence of pCre1 T-DNA (Figure 4B). No hybridisation signal was detected in those lines which had shown no evidence of excision or appeared to be derived from cells which did not contain the pART54 T-DNA. Hybridisation signals were detected in four plants. In three of these plants, 5403T4, 5403T9, and 5420T7, a 1.2 kb hybridising fragment was observed, as would be expected if an intact copy of the pCre1 T-DNA had integrated into the genome. However in 5420T1 a 0.8 kb hybridising fragment was observed suggesting that in this plant a truncated copy of pCre1 T-DNA had been integrated. Southern analysis of *Bam*HI-digested 5420T1 genomic DNA, probed with the 3′ end of the *cre* gene, revealed a hybridisation signal at 0.7 kb (data not shown). This indicated that the position at which integration of pCre1 T-DNA had terminated was within the *ocs* 3′ region, downstream of the *cre* ORF. It seems likely, therefore, that in the 4 lines which showed the presence of pCre1 T-DNA excision was due to expression of *cre* from a stably integrated copy of the recombinase gene.

Lines 5403T8 and 5420T6, which had been shown to have undergone Cre-mediated excision of the *lox*P-flanked region of the pART54 T-DNA, showed no hybridisation to the *cre* probe. This result suggested that in these two plants Cre-mediated excision had been due to transient expression of *cre* recombinase from T-DNA which had been transferred to the plant cell but had not integrated into the genome.

*Bam*HI-digested genomic DNA from lines 5403T2, 5403T4, 5403T8, 5403T9 and 5420T1, 5420T2, 5420T6, 5420T7 was probed with the 1.7 kb *Bam*HI-*Eco*RI *npt*II-*nos*3′ fragment of pART54. A 5.2 kb hybridising fragment was detected in 5403T2, 5420T9 and 5420T2, as expected in lines which had not undergone Cre-mediated excision of the *lox*P-flanked region of the pART54 T-DNA or, as in the case of 5403T9, were chimeric for Cre-mediated excision. No hybridisation to the *npt*II probe was detected in 5403T4, 5403T8, 5420T1, 5420T6, and 5420T7, indicating that in these 5 lines the circular *npt*II-*cod*A molecule, resulting from Cre-mediated excision from the pART54 T-DNA, had not reintegrated elsewhere in the genome (data not shown).

Phenotype screening of T_1 progeny

No seeds from selfed wild-type plants were found to be kanamycin-resistant, whereas the progeny of selfed 5403 and 5420, containing single-copy pART54 T-DNA, showed the expected 3:1 kanamycin-resistant-to-sensitive ratio (Table 2). All of the progeny of selfed 5403T4, 5403T8, 5420T1, 5420T6 and 5420T7, bleached in the presence of kanamycin, confirm-

ing excision of the *npt*II gene. The progeny of 5403T9, which had appeared, by Southern analysis, to be chimeric for excision, showed a 3:1 kanamycin-resistant-to-sensitive ratio, suggesting that the germline of 5403T9 had been derived from cells which had not undergone excision. Likewise, the 3:1 ratio of the progeny of 5420T2 confirmed that this line had not undergone excision of *npt*II.

No progeny of wild-type plants, 5403 or 5420, were found to be resistant to hygromycin (Table 2), whereas 97% and 77% of the 5403T4 and 5420T7 progeny, respectively, were found to be hygromycin-resistant. This suggested that 5403T4 had 2 or 3 unlinked copies of the pCre1 T-DNA integrated into the genome and that 5420T7 had a single pCre1 T-DNA locus. When histochemically stained for β-glucuronidase activity, 26/36 5403T4 and 24/34 5420T7 hygromycin-resistant progeny were found to express the *uid*A transgene, indicating that in both cases the pART54 and pCre1 T-DNAs were unlinked. No progeny of 5403T9 or 5420T1 were found to be resistant to hygromycin. The hygromycin sensitivity of the 5403T9 progeny is consistent with the fact that the germline of this chimeric plant did not contain the pCre1 T-DNA whereas the hygromycin sensitivity of the 5420T1 progeny can be attributed to the premature termination of the pCre1 T-DNA upstream of the *hpt* gene. The absence of hygromycin-resistant progeny of 5403T8 and 5420T6 confirmed the key result that these lines did not contain pCre1 T-DNA and had undergone Cre-mediated excision due to transient expression of *cre*.

In the presence of 5-fc more than 95% of the progeny of wild-type plants and 25% of the progeny of 5403 and 5420 germinated (Table 2). None of these seedlings showed *uid*A expression. For lines 5403T4, 5403T8, 5420T1, 5420T6 and 5420T7, which had undergone excision of *npt*II and *cod*A, 100% of the progeny germinated in the presence of 5-fc and ca. 75% of these seedlings showed β-glucuronidase activity. Of the progeny of 5403T9, 25% germinated in the presence of 5-fc, again consistent with the fact that the germline of this chimeric plant was derived from cells which had not undergone excision and retained the *cod*A gene.

Analysis of 5-fc-tolerant progeny

*Eco*RI/*Eco*RV-digested genomic DNA from four 5-fc-tolerant progeny (a–d) of selfed 5403T4, 5403T8, 5403T9, 5420T1, 5420T2, 5420T6, and 5420T7 was probed with the 3′ end of *uid*A. A 1.9 kb hybridising fragment, indicative of Cre-mediated excision of the *lox*P-flanked region of pART54 T-DNA, was observed in three 5403T4, three 5403T8, three 5420T1, four 5420T6 and four 5420T7 progeny (Figure 5A). The remaining progeny showed no hybridising signal. Only those seedlings with the 1.9 kb hybridising fragment were found to express *uid*A.

The *Eco*RI/*Eco*RV-digested DNA was reprobed with the 3′ end of *cre* (Figure 5B). A 1.2 kb hybridisation signal was detected in all four progeny of 5403T4 and three progeny of 5420T7, indicating that these seedlings contained pCre1 T-DNA. As 5403T4d did not contain the Cre-mediated excision product of the pART54 T-DNA and 5420T7b contained the excision product but not pCre1 T-DNA, this indicated that the pCre1- and pART54-derived T-DNAs had segregated at meiosis. No hybridisation signal was detected in the progeny of 5403T9 which had appeared to be derived from germline cells which did not contain pCre1 T-DNA. A 0.8 kb hybridising fragment was observed in two of the three 5420T1 progeny which contained the pART54 T-DNA Cre-mediated excision product. This indicated that these two progeny contained the truncated version of the pCre1 T-DNA which had integrated into the parental genome. Progeny line 5420T1b showed no pCre1 hybridising signal, indicating segregation at meiosis of the pCre1 and pART54-derived T-DNAs. As expected, no hybridisation signal was detected in the four 5-fc-tolerant progeny of 5420T2, since this line had not been transformed with pCre1. No pCre1 hybridisation signal was detected in the 5-fc-tolerant progeny of 5403T8 or 5420T6, as would be expected if Cre-mediated excision in these lines had occurred as a consequence of transient *cre* expression without pCre1 T-DNA integration.

Discussion

We constructed pART53 for *Agrobacterium*-mediated transformation of plants and subsequent Cre-mediated elimination of the selectable marker genes from transgenic plants. In addition to a kanamycin resistance (*npt*II) gene for the selection of transgenic cells, the T-DNA also contains a 35S transcribed cytosine deaminase (*cod*A) gene. The chimeric *npt*II and *cod*A genes are flanked by *lox*P sequences, in a direct repeat orientation. Therefore in the presence of Cre recombinase this *lox*P-flanked region should be excised from the

Table 2. Phenotype analysis of progeny of selfed pART54 plants which had regenerated in the presence of 5-fc after *A. tumefaciens* (pCre1) cocultivation.

Line	Genotype[1]	Seed viability[2],%	kan^R 250	hyg^R 15	5-fc^R 0.5	GUS^+/5-fc^R
wild type	–	>95	0/107	0/101	52/54	0/26
5403	[*npt*II *cod*A *uid*A]	100	116/146	0/137	30/102	0/30
5403T4	[*uid*A] [*cre-hpt*]	>95	0/140	136/140	130/132	38/50
5403T8	[*uid*A]	100	0/167	0/173	145/147	34/50
5403T9	[*npt*II *cod*A *uid*A] and [*uid*A] [*cre-hpt*]	100	98/131	0/141	53/159	0/46
5420	[*npt*II *cod*A *uid*A]	100	112/145	0/182	23/79	0/23
5420T1	[*uid*A] [*cre*[3]]	100	0/105	0/101	113/113	34/50
5420T2	[*npt*II *cod*A *uid*A]	>97	77/103	0/86	39/127	0/32
5420T6	[*uid*A]	>95	0/91	0/113	127/131	35/50
5420T7	[*uid*A] [*cre-hpt*]	>95	0/157	97/126	123/123	34/48

[1]Genotype based on Southern analysis.
[2]Germination on ½ MS medium.
[3]Truncated pCre1 T-DNA.

plant genome. In this study we cloned a 35S-*uid*A reporter cassette into pART53, to generate pART54, however this can be relatively easily substituted with any gene of interest by insertion into the convenient *Not*I site, which lies outside of the *lox*P-flanked region of the T-DNA.

Expression of the Cre recombinase in transgenic tobacco plants with single-copy pART54 T-DNA inserts and expressing the *npt*II, *cod*A and *uid*A transgenes, by retransformation with pCre1, resulted in precise and efficient excision of the *lox*P-flanked *npt*II-*cod*A region of the T-DNA. This Cre-mediated excision appeared to be independent of the genomic location of the pART54 T-DNA, and occurred irrespective of whether 1 or 2 copies of pCre1 T-DNA were integrated in to the genome. In addition, we observed no evidence of reintegration of the excised molecule elsewhere in the genome. Phenotype screening for kanamycin resistance and 5-fc tolerance of T_1 seeds of selfed T_0 plants confirmed *npt*II and *cod*A excision and indicated that the retransformed lines were homogeneous for excision. It should be noted that although our constructs contained 35S-transcribed *uid*A, *cre*, *hpt* and *cod*A genes, the loss of *npt*II and *cod*A gene expression was not attributable to the gene silencing phenomenon that has been observed previously with genes transcribed from homologous promoters (Vaucheret, 1993).

Through retransformation of pART54 transgenic plants with pCre1 it was possible, therefore, to eliminate the marker genes of the pART54 T-DNA. However, as with other reports on the use of *Cre/lox*P to eliminate marker genes (Dale and Ow, 1991; Russell *et al.*, 1992), this approach introduced a selectable marker gene (*hpt*) linked to the *cre* recombinase. Although the *hpt* marker gene of pCre1 could be segregated away from the pART54 T-DNA at meiosis, to generate plants totally free of marker genes, this need to cross the plants to eliminate all the marker genes offers no advantages over the existing systems in being applicable to producing marker-free perennial fruit crops.

To circumvent the need to cross plants in order to generate marker-free transgenic plants, the possibility of transiently expressing the Cre recombinase from T-DNA was examined. It has been well documented that during *Agrobacterium*-mediated transfer of T-DNA the number of cells to which T-DNA is transferred greatly exceeds, by several orders of magnitude, the number of cells which ultimately become stably transformed and that genes are expressed from the non-integrated T-DNA molecules (Higgins *et al.*, 1992; Janssen and Gardner, 1989). The earliest detection of gene expression from T-DNA encoded genes is 18 h after infection. Expression peaks at 36 h (Narasimhulu *et al.*, 1996) and then declines over 4–10 days (Janssen and Gardner, 1989) as the large numbers of cells which transiently express T-DNA encoded genes fail to become stably transformed.

As higher eucaryotes do not encode a cytosine deaminase we utilised 5-fc and the excision of the conditional lethal dominant *cod*A transgene of the

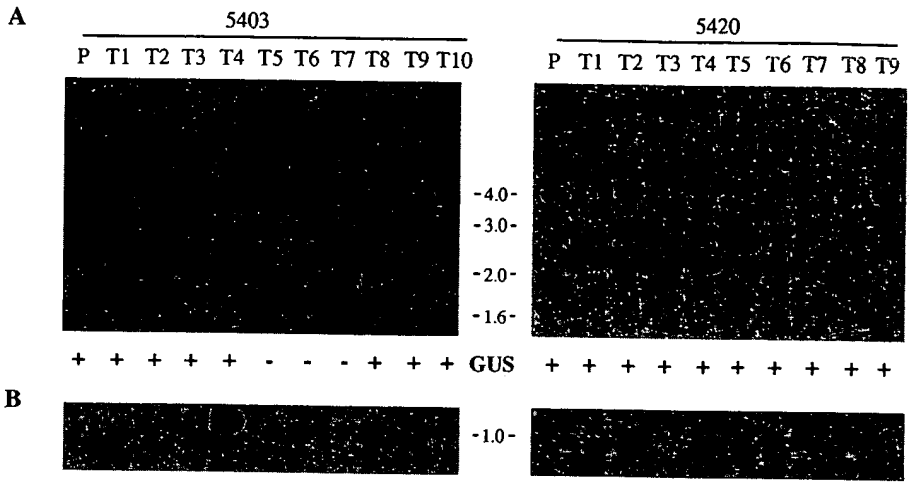

Figure 4. Cre-mediated excision in 5-fc-tolerant pART54 plants regenerated after *A. tumefaciens* (pCre1) cocultivation. **A.** Southern blots of *Eco*RI/*Eco*RV-digested DNA from the parental (P) 5403 and 5420 plants and from ten 5403 (T1–T10) and nine 5420 (T1–T9) derived 5-fc-tolerant plantlets regenerated after *A. tumefaciens* (pCre1) cocultivation. The membranes were probed with a 1.1 kb *Eco*RV/*Bam*HI fragment encoding the 3′ end of the *uid*A gene. Positions of 1 kb markers (Gibco-BRL) and results of histochemical staining of each plantlet for β-glucuronidase are indicated. **B.** Region of the Southern blots described above after reprobing with a 0.5 kb *Eco*RV/*Bam*HI fragment encoding the 3′ end of the *cre* gene. Position of 1 kb marker is indicated.

pART54 T-DNA to select for Cre-mediated recombination events. After cocultivation with *A. tumefaciens* (pCre1) and avoiding the use of hygromycin selection we anticipated that the majority of 5-fc-tolerant shoots regenerated would be derived from cells which had undergone concomitant Cre-mediated excision of *npt*II-*cod*A as a consequence of transient recombinase expression, without T-DNA integration. Of the shoots derived from two independent pART54 transformed lines 2.2% and 2.8% were found to show tolerance to 5-fc, although not all of these shoots appeared to grow as healthy plantlets until they were removed from 5-fc.

Molecular analysis revealed that three shoots (derived from line 5403) survived the 5-fc treatment as they appeared to lack the pART54 T-DNA, suggesting that the 5403 line was chimeric. Several reports have previously described the occurrence of chimeric transgenic tobacco plants (Oono *et al.*, 1993; Schmulling and Schell, 1993). Ten shoots which survived the 5-fc treatment were found not to have undergone Cre-mediated recombination and therefore retained the *cod*A gene. Generally, these shoots showed a degree of chlorosis in the presence of 5-fc. The molecular analysis did however reveal that six shoots were tolerant to 5-fc as a consequence of Cre-mediated excision of the *cod*A gene. Only one of these six shoots showed a degree of chlorosis in the presence of 5-fc and was probably a consequence of this line being chimeric for excision of the *npt*II-*cod*A region. It is our opinion that the use of this negative selection was critical to the isolation of plantlets which had undergone the relatively rare Cre-mediated excision events. Although it appears that at the levels of 5-fc used in this study expression of *cod*A did not provide a completely clean negative-selection system it did provide a method of enrichment for plantlets derived from cells which had undergone Cre-meditated excision. Perhaps a cleaner selection could be achieved by using higher 5-fc levels and a more rigorous selection of normal healthy plantlets. The use of *cod*A as a negative-selectable marker has been described in lotus, tobacco, and *Arabidopsis* (Perera *et al.*, 1993; Stougaard, 1993) and there is no reason to suspect that it will not be applicable to the majority of higher-plant species.

As Southern analysis revealed that 6 of 19 shoots from two independent parental lines were tolerant to 5-fc as a consequence of Cre-mediated excision of the *cod*A gene, this indicated that 0.7–0.8% of the shoots had regenerated from cells to which the pCre1 T-DNA had been transferred and the *cre* recombinase had been expressed. Enhancing the virulence of *Agrobacterium* during cocultivation, by the addition of acetosyringone (Stachel *et al.*, 1985), could be used to increase the number of plant cells to which the pCre1 T-DNA is transferred. This may increase the percentage of plantlets regenerated from cells that have undergone Cre-mediated excision. What was a rather unexpected finding was that in four of the six plantlets that had

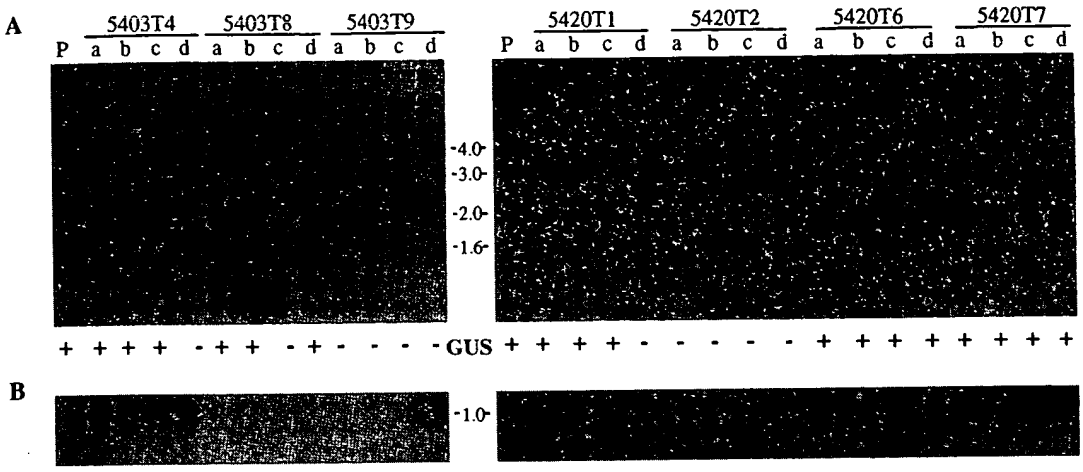

Figure 5. 5-fc-tolerant progeny of selfed pART54 plants regenerated after *A. tumefaciens* (pCre1) cocultivation. Southern blots of *Eco*RI/*Eco*RV-digested DNA from 5-fc-tolerant progeny (a–d) of selfed lines 5403T4, T8 and T9 and 5420T1, T2, T6 and T7 and from selfed parental lines 5403 and 5420 (P) (not 5-fc-selected). The membranes were probed with a 1.1 kb *Eco*RV/*Bam*HI fragment encoding the 3′ end of the *uid*A gene. Positions of 1 kb markers (Gibco-BRL) and results of histochemical staining of each seedling for β-glucuronidase are shown. **B.** Region of the Southern blots described above after reprobing with a 0.5 kb *Eco*RV/*Bam*HI fragment encoding the 3′ end of the *cre* gene. Position of 1 kb marker is indicated.

undergone Cre-mediated excision, pCre1 T-DNA had actually been integrated into the genome. Thus despite the absence of hygromycin selection for pCre1 integration, ca. 0.5% of the regenerated shoots were derived from cells which had become stably transformed with pCre1 T-DNA. This frequency may in fact be higher, since we employed a 5-fc selection for *cre* recombinase expression and recombination. We also observed in one of the four instances of pCre1 T-DNA integration that there appeared to be premature termination of T-DNA integration such that the recombinase was integrated into the genome but the region distal to the right T-DNA border, including the *hpt* gene, was not. Truncation of the T-DNA is not an uncommon occurrence during *Agrobacterium*-mediated transformation (Deroles and Gardner, 1988; Gheysen *et al.*, 1990). In this instance truncation of the pCre1 T-DNA at a position upstream of the *hpt* gene, coupled with Cre-mediated excision of the *lox*P-flanked region of the pART54 T-DNA, resulted in this line being totally free of marker genes, albeit fortuitously.

Progeny analysis of the lines which had pCre1 T-DNA integrated into the genome confirmed the excision of the *npt*II and *cod*A marker genes and integration of the *cre*-linked *hpt* marker. Progeny were also identified where the remaining *uid*A transgene of the pART54 T-DNA and the pCre1 T-DNA had segregated at meiosis, generating marker-free transgenic plants. The use of 5-fc proved to be extremely effective in preventing the germination of plants which retained *cod*A transgene expression and this perhaps could be utilised to screen the progeny of large numbers of plants to identify those which have undergone Cre-mediated excision.

Two 5-fc-tolerant regenerated shoots were identified as having undergone Cre-mediated excision of the *lox*P-flanked region of pART54 without integration of the pCre1 T-DNA. It seems highly probable that in these two instances excision was a consequence of transient expression of the recombinase from the pCre1 T-DNA. Due to the orientation of the *cre* gene in pCre1 relative to the right T-DNA border, the coding strand of the *cre* gene would be transferred to the plant as the T-strand. Therefore, this single-stranded T-strand must have been converted to a double-stranded form to provide the template for *cre* transcription. The Cre-mediated recombination event must take place efficiently and relatively early in the developing shoots given that transient expression from the T-DNA occurs for only a limited time period (Janssen and Gardner, 1989; Narasimhulu *et al.*, 1996). Our findings also suggested that the 35S promoter gave sufficient transient expression of *cre* in the progenitor cell type to result in the occurrence of Cre-mediated recombination events.

Using transient expression of the recombinase and 5-fc selection of concomitant excision of the *cod*A and *npt*II genes we achieved our aim of obtaining trans-

genic plants completely free of marker genes without the need to sexually cross plants. The absence of marker genes was confirmed by the sensitivity of T_1 seeds of selfed-T_0 plants to either kanamycin or hygromycin. This phenotype testing also confirmed that the plants were homogeneous for excision.

It was somewhat surprising to find that Cre-mediated excision could be attributed to expression of the recombinase from stably integrated copies of pCre1 in more of the 5-fc-tolerant shoots than could be attributed to transient expression, without T-DNA integration. This may have been a consequence of the high efficiency of *Agrobacterium*-mediated transformation of tobacco. In plant species which are not as readily stably transformed as tobacco it appears that T-DNA transfer and the expression of genes from T-DNA molecules does occur at a high frequency and that it is T-DNA integration into the genome that is limiting (Narasimhulu *et al.*, 1996). This being the case it is possible that the approach of transiently expressing the recombinase from unintegrated T-DNA molecules and avoiding T-DNA integration could be even more amenable to such plant species.

In conclusion, we have shown that transgenic plants can be produced which are entirely free of selectable marker genes and that this can be achieved without sexual crossing. Our approach of transiently expressing the *cre* recombinase via *Agrobacterium*-mediated T-DNA transfer and utilising a conditional lethal dominant marker to enrich for plants which are marker-free should be applicable to many of the established perennial horticultural cultivars requiring vegetative propagation to maintain their elite genotype.

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